# Binding of Heparin by Type III Domains and Peptides from the Carboxy Terminal Hep-2 Region of Fibronectin†

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*Received June 17, 1993; Revised Manuscript Received September 7, 1993*®

ABSTRACT: The major sites of heparin binding by fibronectin are located in fragments of 30 or 40 kDa that contain type III modules 12 through 14 or 15. Various proteolytic or recombinant subfragments and several synthetic peptides derived from this region have been compared with respect to their binding to fluorescein-labeled heparin in solution. Binding was monitored by the change in fluorescence anisotropy at 25 °C and pH 7.4 in 0.02 M Tris buffer, alone (TB) or with 0.15M NaCl (TBS). A 23-kDa fragment containing $III_{13}$ and $III_{14}$ but lacking $III_{12}$ had $K_d$ values of 0.3 and 1.8 $\mu$M in TB and TBS, respectively, indistinguishable from the 30-kDa parent. Fragments containing only module $III_{13}$ bound 2–3-fold weaker than the parent while those containing only $III_{14}$ bound 6–50-fold weaker depending on the ionic strength. Fragments containing only $III_{12}$ or $III_{15}$ failed to bind at all in TBS. A cationic peptide derived from the amino terminus of $III_{13}$ and containing the Arg-Arg-Ala-Arg consensus sequence, whose integrity was shown by Barkalow and Schwarzbauer [Barkalow, F. J., & Schwarzbauer, J. E. (1991) *J. Biol. Chem. 266*, 7812–7818] to be critical, failed to bind in TBS but bound weakly in TB. Two additional cationic peptides derived from the middle and C-terminal regions of $III_{14}$ showed similar behavior. Thus while the major determinant(s) of heparin binding are located in $III_{13}$, those determinants are only active when part of a properly folded structure. Furthermore, module $III_{13}$ when isolated had a slightly lower affinity than fragments containing both $III_{13}$ and $III_{14}$. It is concluded that interactions between these two modules may be important to arrange positively charged residues from both modules for optimal recognition by heparin.

Fibronectin is a large glycoprotein which occurs on cell surfaces, in the connective tissue matrix, and in extracellular fluids (Hynes, 1990). It consists of two very similar subunits of molecular mass 250 000 daltons, held together at the C-terminal region by disulfide bonds. Each subunit contains numerous homologous repeats or modules, of three different types (Skorstengaard et al., 1986). These are gathered in groups to form functional domains which are specialized for binding integrin receptors and other macromolecules such as fibrin, collagen, and heparin. The latter is a sulfated glycosaminoglycan analogous to those found on cell surfaces and in the extracellular matrices of a wide variety of animal tissues (Lindahl & Kjellén, 1991). The heparin-binding region of fibronectin is thought to interact with cell surface glycosaminoglycans to facilitate cell adhesion and spreading (Izzard et al., 1986; LeBaron et al., 1988; McCarthy et al., 1988; Lewandowska et al., 1987; Woods et al., 1986; Jalkanen & Jalkanen, 1992). This region also contains a sequence that mediates the adhesion of human melanoma cells and some hemopoietic cells via the $\alpha_4\beta_1$ integrin receptor (Mould & Humphries, 1991; Sanchez-Aparicio et al., 1993).

The interaction of heparin with fibronectin is dominated by the so called hep-2 domains located in the C-terminal third of each polypeptide chain (Yamada et al., 1980; Hayashi & Yamada, 1982; Benecky et al., 1988; Ingham et al., 1990; Barkalow & Schwarzbauer, 1991). Functionally active fragments containing these domains can be isolated from proteolytic digests of the parent molecule. The 30-kDa hep-2A fragment, obtained from the heavy chain, contains three type III modules 12–14 whereas the 40-kDa hep-2B fragment contains four such modules ($III_{12}$–$III_{15}$). Titration experiments showed that these two fragments were indistinguishable with respect to their binding affinities for fluorescent labeled heparin, indicating that $III_{15}$ was unimportant (Ingham et al., 1990). More recently, Barkalow and Schwarzbauer (1991) used recombinant methods to examine the effects of deleting specific portions of the hep-2 region on the ability of the resulting variants to bind heparin-Sepharose. That work indicated that a specific region near the N-terminus of $III_{13}$ was critical for binding to heparin-Sepharose. Additional evidence for a critical role of $III_{13}$ comes from Kimizuka et al. (1991), who found that recombinant derivatives from which $III_{12}$ or $III_{14}$ were deleted appeared to bind immobilized heparin whereas those lacking $III_{13}$ did not. Although binding constants were not determined in either study, the results of Barkolow and Schwarzbauer suggested that $III_{14}$ was required for full activity.

Deletion or mutation of amino acid residues or whole modules could affect binding properties in two ways: directly, if the deleted residues are involved in contacts with the ligand, or indirectly by altering the ability of the expressed mutants to properly fold or by perturbing the conformation of neighboring structures that are important for binding. A study of the domain structure and stability of the hep-2 regions of fibronectin was recently reported from this laboratory (Novokhatny et al., 1992). It was found that type III modules 12–15 constitute independently folded domains and that modules 13 and 14 strongly interact with each other. It was suggested that these domain–domain interactions might be important for holding positively charged residues in proper orientation for optimum recognition by heparin. Here we

† Supported in part by National Institutes of Health Grants HL21791 & HL44336.

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® Abstract published in *Advance ACS Abstracts*, November 1, 1993.

0006-2960/93/0432-12548$04.00/0 

examine the interaction of heparin with various hep-2 subfragments whose folding status was established in the previous study, as well as a recombinant fragment comprising module $III_{13}$. In addition to testing the ability to bind to immobilized heparin, we have measured the dissociation constants for binding to fluorescent-labeled heparin in the fluid phase, providing a more direct and quantitative comparison. The results confirm the primary role of module $III_{13}$ in the interaction and establish the necessity of module $III_{14}$ for full activity. Measurements with synthetic peptides reveal the importance of tertiary structure in defining the active site(s).

## MATERIALS AND METHODS

Thermolysin, TPCK trypsin, pepsin, pepstatin, phenylmethanesulfonyl fluoride (PMSF), and porcine mucosal heparin (sodium salt) were obtained from Sigma Chemical Co. (St. Louis, MO). Endopeptidase Lys C was from Boehringer Mannheim GmbH (Indianapolis, IN). All other chemicals were reagent grade or better.

Fluorescein-labeled heparin was prepared by the method of Ogama et al. (1982) and characterized as previously described (Ingham et al., 1990). All experiments described here were performed with Sephadex G-100 fraction no. 4 having an average molecular weight of ~15 000 daltons (Ingham et al., 1990). Heparin-Sepharose was prepared using unbleached bulk porcine mucosal heparin as described (Ingham et al., 1990).

Human plasma fibronectin (Fn) was isolated as described by method A of Miekka et al. (1982). The 30- and 40-kDa hep-2 fragments were isolated from a thermolysin digest of Fn as previously described (Borsi et al., 1986; Ingham et al., 1990). The various fragments and subfragments are illustrated schematically in Figure 1 where the first number preceding the K in the designation of each subfragment indicates the apparent $M_r$ by SDS–PAGE, and the number(s) following the K indicate the composition in terms of type III modules. Most subfragments were generated by digestion of 30K or a mixture of 30K and 40K with trypsin at an enzyme/substrate ratio of 1:100 for 8 h at 37 °C in TB [0.02 M tris(hydroxymethyl)aminomethane containing 0.02% $NaN_3$ at pH 7.4]. PMSF was used to stop the reaction. The 10K12 fragment was generated from 30K with pepsin at pH 2.8 in 0.05 M glycine buffer at an enzyme/substrate ratio of 1:50 for 12 h at 35 °C. The reaction was stopped by adding pepstatin and raising the pH to 7.4 with tris(hydroxymethyl)-aminomethane base. The 23K13–14 fragment was generated by digestion of 10 mg of 30K at 37 °C in 5 mL of TBS (TB + 0.15 M NaCl) with a total of 20 μg of endopeptidase Lys-C added in 5-μg amounts at 18-h intervals. The 14K13 fragment was obtained in low yield by digestion of 23K (0.4 mg/ml in 0.1 M sodium-acetate, pH 3.05) with pepsin (0.05 mg/mL) at 37 °C for 20 h or by digestion of 30K (2 mg/mL in 0.05 M glycine, pH 2.8) with pepsin (40 μg/mL) for 12 h at 35 °C. The various digestion mixtures were fractionated by affinity chromatography on heparin-Sepharose in TB eluting with a linear gradient of NaCl. The subfragments were further purified by size-exclusion and/or ion exchange chromatography as required to achieve homogeneity as defined by SDS–PAGE and sequencing. For instance, the 20K14–15 is the only bimodular fragment that survives trypsin digestion and was easily separated from smaller fragments by size-exclusion chromatography on Bio-gel P-60 (Bio-Rad). The 8K15 fragment was separated from other fragments of similar size by virtue of its ability to bind DEAE-Sephacel (Pharmacia).

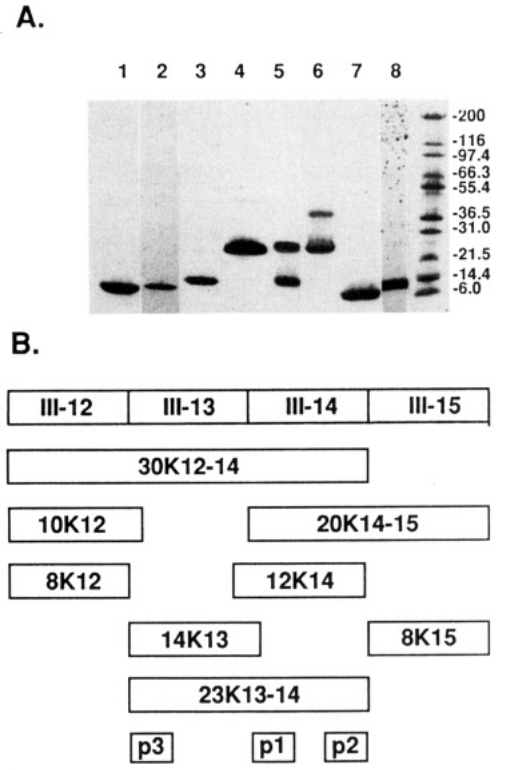


FIGURE 1: (A) SDS–PAGE of subfragments used in this study. Lanes 1–7 represent the various fragments arranged according to their amino termini beginning with 8K12 and followed by 10K12, 14K13, 23K13–14, 12K14, 20K14–15, and 8K15. Lane 8 contains recombinant 9.5K13, and the far right lane contains molecular weight standards with the indicated masses in kilodaltons. The samples shown here were not reduced before electrophoresis; essentially identical results were obtained with reduced samples. (B) Schematic illustration of the location of the various fragments and peptides within the 40K hep-2B fragment (top). Numbers on the left of the K indicate the approximate molecular mass in kDa, and those on the right indicate the composition in terms of type III modules.

The identities of all fragments were confirmed by sequencing the first few residues with a Hewlett Packard G1000S protein sequencing system [see Table I for the identification of the $NH_2$ termini, based on the numbering system of Skorstengaard et al. (1986)]. With the exception of 14K13, all fragments displayed a single sequence. The 14K13 fragment was actually a mixture of three similar fragments with amino termini at $Thr^{1689}$ (~25%), $Glu^{1691}$ (50%), and $Asn^{1692}$ (25%). Amino acid analysis indicated that 10K12 extended ~17 residues into module $III_{13}$, terminating after $Arg^{1700}$ and before $Ile^{1709}$. With the exception of those containing module $III_{14}$, all fragments were homogeneous by SDS–PAGE in 8–25% gradient acrylamide precast gels (Pharmacia Phast system). As shown in Figure 1, 23K13–14 (lane 4), 20K14–15 (lane 5), and especially 12K14 (lane 6) had a tendency to form dimers during electrophoresis in SDS. The dimerization appears to be an artifact of SDS–PAGE since all fragments were very homogeneous by size-exclusion chromatography on Superdex-75 (Pharmacia) and eluted in a position consistent with their size (Figure 2). The dimerization phenomenon was observed in both nonreducing and reducing conditions and was not related to a similar phenomenon sometimes seen with fragments that contain module $III_{15}$ which has a cryptic free sulfhydryl group that becomes exposed in SDS (Wagner & Hynes, 1979) and in our hands tends to form reducible dimers unless an alkylating agent is added to the nonreducing SDS sample buffer.

Recombinant 30K12–14 and 9.5K13 were produced as fusion proteins with maltose binding protein in *Escherichia*

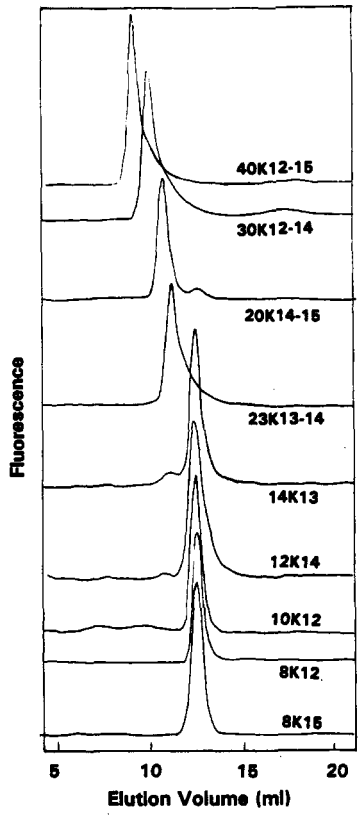


FIGURE 2: Size-exclusion chromatography of the fragments used in this study. Analytical amounts of the fragments were chromatographed on a Superdex-75 column at room temperature in 0.02 M Tris-HCl, pH 7.4, 0.02% $NaN_3$, and 0.15 M NaCl (TBS). Elution was monitored by intrinsic fluorescence at 350 nm. Tracings of the observed profiles have been shifted vertically to improve visibility.

*coli* using the pMAL-p2 expression vector (New England Biolabs). cDNA fragments were prepared by PCR using synthetic primers flanking the desired regions and containing the appropriate restriction sites for ligation into the vector. After digestion with restriction enzymes, the vector and cDNA fragments were purified by agarose electrophoresis, ligated and used to transform TB1 *E. coli* cells by electroporation. The cells were grown on an LB agar plate containing 50 μg/mL ampicillin, 40 μg/mL X-gal, and 40 μg/mL IPTG. The transformed white cells were used to inoculate liquid LB media containing 50 μg/mL ampicillin, grown overnight at 37 °C with shaking, and diluted 100-fold with fresh ampicillin-containing media. When the optical density reached ~0.6 at 600 nm, IPTG was added to 0.3 mM and incubation continued for an additional 3 h. The cells were harvested by centrifugation and lysed by sonication in 0.005% Triton X-100, and the fusion protein was purified by affinity chromatography on immobilized amylose and/or heparin. After cleavage with factor Xa, the desired products were isolated by rechromatography on the same columns and further purified on QMA anion exchange media (Waters). Both recombinant fragments were homogeneous by SDS–PAGE and size-exclusion chromatography, had the expected N-terminal sequences, and exhibited reversible melting transitions similar to those obtained with their natural counterparts (Novokhatny et al., 1992).

Figure 1 also illustrates the locations of three synthetic peptides with the following definitions:

pep-1, K20V, $Lys^{1815}$ to $Val^{1834}$ (KYEKPGSPPREVVPRPRPGV)

pep-2, K15T, $Lys^{1856}$ to $Thr^{1870}$ (KNNQKSEPLIGRKKT)

pep-3, N19T, $Asn^{1692}$ to $Thr^{1711}$ (NVSPPRRARVTDATETTIT)

Peptides were synthesized on a MilliGen model 9050 peptide synthesizer using Fmoc chemistry with pentafluorophenyl amino acid active esters. A polyamide Kieselguhr support was used for peptides 1 and 2 to produce a C-terminal carboxylic acid while a polystyrene PAL [5′-(4′-(fluorenylmethoxycarbonyl)aminomethyl-3′,5′-dimethoxyphenoxy)-valeric acid] linker was used for peptide 3 to produce a C-terminal amide. All peptides were purified to homogeneity on a reverse-phase C-18 column. Their integrity was confirmed by amino acid analysis, which was also used to determine the concentrations of their stock solutions.

Concentrations of the fragments were determined from the absorbance at 280 nm, using molar extinction coefficients (ε, $M^{-1}$ $cm^{-1}$) calculated by the method of Edelhoch (1967) from the amino acid sequence data of Skorstengaard et al. (1986) and Kornblihtt et al. (1985). The values of ε are 30K, 30 216; 23K13–14, 21 620; 8K12 and 10K12, 9530; 14K13, 10 810; 12K14, 10 810; 20K14–15, 19 060; 8K15, 8250.

Analytical affinity chromatography utilized the Pharmacia FPLC system and a 2.7-mL column of heparin-Sepharose equilibrated at low ionic strength in TB at a flow rate of 1.0 mL/min. Bound proteins were eluted with a linear gradient of NaCl, with monitoring of either the absorbance at 280 nm or the intrinsic fluorescence. Analytical size-exclusion chromatography was performed with the same system using a Superdex-75 column.

Fluorescence anisotropy measurements were made with an SLM-8000C spectrofluorometer in the T format with excitation and emission wavelengths of 493 and 524 nm, respectively. Titrations of 0.1 μM fluorescein-labeled heparin in TB or TBS were performed by manual addition of small amounts of a stock solution of the fragment or, in the case of recombinant fragments and synthetic peptides, by continuous addition with a motorized syringe controlled by the same computer that controls the fluorometer. The change in anisotropy, $\Delta A$, as a function of titrant concentration was fitted to a single class of equivalent binding sites on the fragment by using the following equation:

$$\Delta A = \Delta A_{max}[\text{titrant}]/(K_d + [\text{titrant}]) \quad (1)$$

where [titrant] is the free concentration of fragment (or peptide), $\Delta A_{max}$ is the maximum anisotropy change that would be produced at saturating concentrations of titrant, and $K_d$ is the apparent dissociation constant of the heparin-fragment complex. Since in all cases the concentration of FA-heparin was low compared to the range of concentration of fragment, the free fragment concentration was taken as the total.

Unless otherwise indicated, experiments were performed in 0.02 M tris(hydroxymethyl)aminomethane (Tris) buffer, pH 7.4, containing 0.02%$NaN_3$ and no NaCl (TB) or 0.15 M NaCl (TBS).

## RESULTS

*Affinity Chromatography on Heparin-Sepharose.* The 30- and 40-kDa fragments and their subfragments and synthetic peptides were applied to heparin-Sepharose in TB and eluted with a linear gradient of NaCl. The concentrations of NaCl required for peak elution are summarized in column 4 of Table I. All but one of the fragments bound quantitatively under these low ionic strength conditions. Fragment 8K15 represents

Table I: Heparin Binding Properties of Hep-2 Fragments and Subfragments of Fibronectin

| name of fragment | enzyme | amino terminus | [NaCl][a] | $K_d$ (μM) (TB)[b] | $K_d$ (μM) (TBS)[b] |
|---|---|---|---|---|---|
| 40K12–15 | thermolysin | Ala-1597 | 0.47 | nd | |
| 30K12–14 | thermolysin | Ala-1597 | 0.49 | 0.35 | 2.1–2.8 |
| r30K12–14 | recombinant | Ala-1597 | 0.52 | nd | 2.8 |
| 23K13–14 | Lys-C | Asp-1676 | 0.51 | 0.35 | 1.8 |
| 14K13 | pepsin | Glu-1691 | 0.43 | 0.83 | 8.4 |
| r9.5K13 | recombinant | ILN-1692[c] | 0.50 | 0.52 | 4.8–7.2 |
| 20K14–15 | trypsin | Ser-1771 | 0.10 | 2.4 | >100 |
| 12K14 | trypsin | Ser-1771 | 0.11 | 5.1 | 90 |
| 8K12 | trypsin | Ala-1597 | 0.034 | 16.5 | >100 |
| 10K12 | pepsin | Ala-1597 | 0.067 | 3.5 | >100 |
| 8K15 | thermolysin | Thr-1870 | no binding | >100 | >100 |
| peptides | | | | | |
| 1 (K20V) | synthetic | Lys-1815 | 0.10 | 52 | no bind |
| 2 (K15T) | synthetic | Lys-1856 | 0.16 | 9.0 | 290 |
| 3 (N19T) | synthetic | Asn-1692 | 0.12 | 41 | no bind |

[a] Concentration of NaCl for peak elution from heparin-Sepharose. [b] TB = Tris buffer without NaCl; TBS contains 0.15 M NaCl. [c] Contains an Ile-Leu sequence in addition to module $III_{13}$ beginning at Asn-1692.

the 15th type III module which is not capable of binding to heparin by itself. Fragment 23K13–14 contains complete copies of modules 13 and 14 and is seen to require as much NaCl for elution as the parent fragments or r30K. The single-module fragments require widely different ionic strengths for elution. Fragment 14K13 and r9.5K13 contain intact module $III_{13}$ and elute slightly earlier than the respective 30K parents. Fragment 12K14 elutes below physiological ionic strength, at a position similar to that of fragment 20K14–15 which also contains module $III_{14}$. Fragment 8K12 contains module $III_{12}$ and elutes at very low ionic strength. The slightly stronger binding of fragment 10K12 could be due to the fact that it extends ~17 residues into the N-terminus of $III_{13}$, a region identified as critical by Barkalow and Schwarzbauer (1991). Thus, the 13th and 14th type III modules alone appear by this criterion to have an affinity for heparin that is intermediate between that of fragments that contain both of these modules and fragments that lack either of them. When *applied* at physiological ionic strength, the only subfragments binding to the column were 23K13–14, 14K13, and r9.5K13.

All three synthetic peptides bound to some degree when small amounts were applied to the heparin-Sepharose column in TB but all eluted near or below physiological ionic strength. When applied at physiological ionic strength, none of the peptides bound.

*Titration of Fluorescent-Labeled Heparin with Fragments.* When FA-heparin is titrated with 30K or 40K fragments, it undergoes a large increase in fluorescence anisotropy that can be completely reversed with unlabeled heparin (Ingham et al., 1990). Figure 3 compares titration data for several subfragments with those of the 30K parent fragment in 0.02 M Tris buffer, pH 7.4, in the absence (panel A) and presence (panel B) of 0.15 M NaCl. Smooth curves represent best fits of the data to eq 1, providing the apparent $K_d$ values summarized in Table I. The observed pattern is for the most part analogous to that seen by affinity chromatography. The 23K13–14 fragment binds with a $K_d$ that is very close to that of the 30K parent, both in the presence and absence of NaCl; the $K_d$ is ~5-fold higher at physiological ionic strength. Fragment 14K13 had the lowest $K_d$ of any of the single module fragments and was the only single-module fragment derived by proteolysis that caused a substantial increase in anisotropy at physiological ionic strength. Fragments lacking intact $III_{13}$ had negligible effects on the anisotropy at physiological ionic

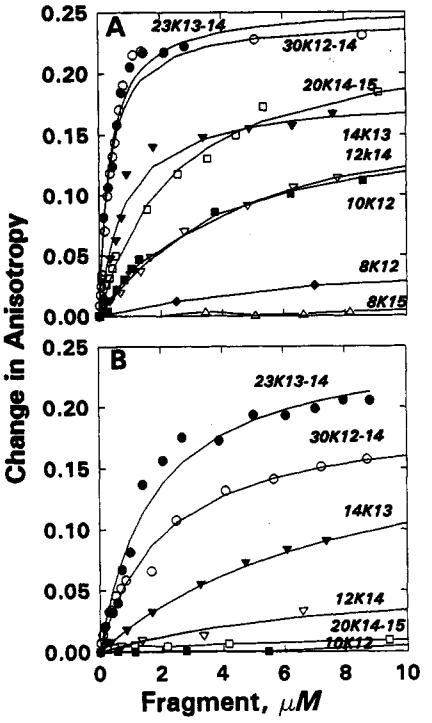


FIGURE 3: Titration of FA-heparin (0.1 μM) with hep-2 fragments and subfragments while monitoring the anisotropy of the fluorescein fluorescence at 25 °C. (Panel A) 0.02 M Tris-HCl pH 7.4, 0.02% $NaN_3$ (TB). (Panel B) Same as panel A but with 0.15 M NaCl (TBS). Solid lines represent best fits of the data to eq 1. The corresponding values of $K_d$ are given in Table I.

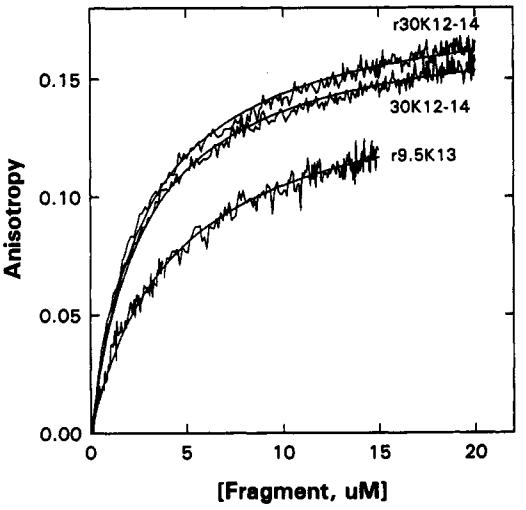


FIGURE 4: Titration of FA-heparin (0.1 μM) with natural and recombinant 30K hep-2A fragments and recombinant 9.5K13 fragment in TBS at 25 °C. The solid lines represent best fits of the data to eq 1 to obtain the $K_d$ values in Table I.

strength but some of them bound significantly in the absence of salt. Fragment 10K12 which extends into $III_{13}$ had a $K_d$ ~5-fold lower than the shorter 8K12 at low ionic strength. Fragment 8K15 gave no response under either condition.

Figure 4 presents titration data obtained in TBS with the recombinant fragments, r30K12–14 and r9.5K13. The former is seen to produce a change in anisotropy and a $K_d$ that is indistinguishable from that of its natural counterpart, which is included for comparison. The smaller r9.5K13 produces a slightly smaller change in anisotropy and has an apparent $K_d$ about 2-fold higher than the larger fragment.

*Interaction of Fluorescent-Labeled Heparin with Peptides.* The ability of several synthetic peptides to compete with 30K

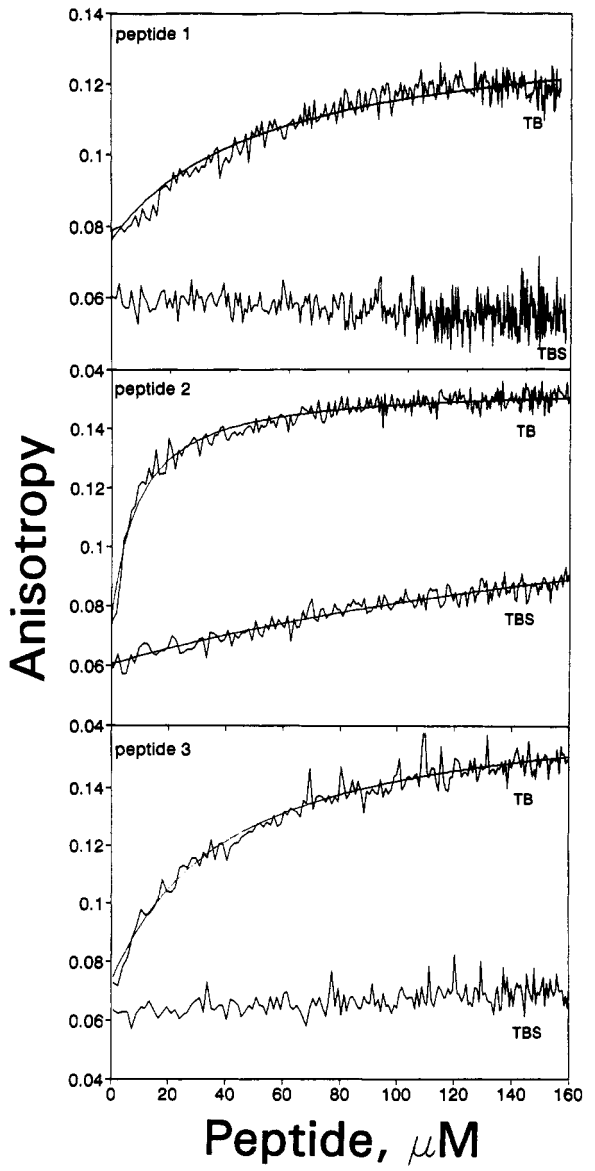


FIGURE 5: Titration of FA-heparin (0.1 $\mu$M) with peptides in 0.02 M Tris-HCl, pH 7.4, 0.02% $NaN_3$ in the absence (TB) and presence (TBS) of 0.15 M NaCl at 25 °C. The solid curves represent best fits of the data to eq 1 to obtain the $K_d$ values in Table I.

for binding to FA-heparin was examined in TBS. It was expected that high concentrations of active peptides might diminish the increase in anisotropy caused by 30K by competing with it for binding to the heparin molecule. Since the peptides are much smaller than 30K, they would not be expected to enhance the anisotropy nearly as much as the larger fragment, if at all. Peptides were added to a solution of FA-heparin which already contained enough 30K to give a $\Delta A$ that was ~75% of maximal. None of the peptides caused a measurable decrease in the anisotropy at concentrations up to 150 $\mu$M (not shown).

The results of direct titrations of FA-heparin with peptides 1,2, and 3 are shown in Figure 4. All three peptides caused hyperbolic changes in anisotropy at low ionic strength (TB). The apparent $K_d$s ranged between 9 and 52 $\mu$M, with peptide 2 having the highest affinity. Peptide 2 was the only one to cause a detectable change in anisotropy at physiological ionic strength (TBS) where it bound weakly with an apparent $K_d$ of 290 $\mu$M. These direct titration results explain the inability of these peptides to significantly inhibit the binding of the 30K fragment with FA-heparin in TBS.

## DISCUSSION

The 30- and 40-kDa fragments are readily cleaved with a variety of enzymes to yield subfragments containing intact type III modules. These were analyzed with respect to their ability to bind heparin Sepharose and enhance the fluorescence of fluoresceinamine-labeled heparin. The former is a non-equilibrium solid-phase assay that provides only a quantitative comparison whereas the latter provides a quantitative estimate of the dissociation constant. Since the concentration of labeled heparin was negligible compared to the concentration of fragment required to increase the anisotropy, the titrations provide no information regarding stoichiometry. It is reasonable to suspect that a single heparin molecule could bind more than one molecule of fragment. However, the dose–response is reasonably well described by a single class of sites suggesting the absence of heterogeneity or cooperativity between sites. In a previous study it was shown that the anisotropy response could be completely reversed by addition of unlabeled heparin indicating that the presence of the dye does not strongly influence the interaction.

Removal of module $III_{12}$ from the 30K parent fragment to produce the 23K lys-C fragment had no effect on the dissociation constant, and the 8K fragment containing only this module showed no detectable binding to heparin at physiological ionic strength. The same is true for module $III_{15}$ which is present in the larger 40-kDa hep-2B fragment derived from the light chain of Fn. Module $III_{15}$ is highly acidic with a calculated pI of 4.7 and thus would not be expected to bind to heparin. Modules 12, 13, and 14 are all highly basic with calculated pI values of 10.0 ± 0.1, a little higher than the measured value of 9.3 for the parent 30K fragment (Borsi et al., 1986). Thus, while the interaction is clearly electrostatic in nature (Novokhatny et al., 1992), the negligible binding of module $III_{12}$ cannot be attributed to a lack of net positive charge. Rather, it is probably the arrangement of that charge in space that governs affinity for heparin (Rovelli et al., 1992).

All of the natural fragments used here have been shown in a separate study to retain a compact folded structure which exhibits a cooperative unfolding transition when subjected to heat or chemical denaturants (Novokhatny et al., 1992). The same is true for the recombinant fragments (not shown: see Materials and Methods). Each of the type III modules forms an independently folded domain that retains its structure upon isolation. The important heparin-binding determinants appear to be localized in modules $III_{13}$ with a small contribution from $III_{14}$. The calorimetric measurements indicate a strong interaction between these two modules that persists even in 6 M urea where heparin still binds (Novokhatny et al., 1992). This interaction may be important for maintaining the spatial relationship between positively charged residues that participate in heparin binding. Module $III_{13}$ contains an RRAR sequence near its amino terminus, and module $III_{14}$ contains an RKK triplet near its C-terminus. The former sequence fits one of the heparin-binding consensus patterns proposed by Cardin and Weintraub (1989), perhaps accounting for the higher affinity of $III_{13}$. Barkolow and Schwarzbauer (1991) showed that deletion of a short region containing most of that consensus sequence, or mutation of two arginines within that sequence, essentially abolished the ability of recombinant fibronectin derivatives to bind to heparin-Sepharose at physiological ionic strength. This same consensus sequence is in the C-terminal region of fragment 10K12, which extends ~17 residues into module $III_{13}$. Although this fragment bound at low ionic strength with higher affinity than 8K12, which

lacks this sequence, its binding was much weaker than that of fragments 14K13 and r9.5K13, which contain this sequence in a properly folded module. Peptide 3, which also contains this RRAR sequence, bound only weakly at low ionic strength and not at all at physiological ionic strength, further emphasizing the importance of tertiary structure. It is unlikely that the peptide or the 17 residue extension on 10K12 has the same conformation as in native $III_{13}$. The effects of the above-mentioned deletions or mutations on the conformation of module $III_{13}$ are not known. The results with our r9.5K13 fragment prove by direct measurement that most of the binding ability of the hep-2 region of Fn resides in module $III_{13}$.

According to McCarthy et al. (1988), peptides 1 and 2, derived from $III_{14}$, when coated on plastic, are able to capture measurable amounts of $^3$[H]heparin. In our hands, these peptides, like the module from which they were derived, bound to heparin-Sepharose only at low ionic strength. When used to titrate FA-heparin in the fluid phase, the highest affinity was obtained with peptide 2 which comes from the C-terminus of $III_{14}$ and contains a triple basic RKK sequence. This was the only peptide to display measurable affinity at physiological ionic strength. Thus, if one were to base conclusions on data derived with peptides alone, one would be under the erroneous impression that module $III_{14}$ was the major source of heparin-binding activity. Module $III_{14}$ binds much more weakly than module $III_{13}$ but when attached to the latter is able to enhance the affinity to a level equal to that of the parent fragment.

In summary, the results presented here indicate that isolated type III modules 12–14 have differing affinities which, depending on the ionic strength, are 2- to more than 100-fold weaker than that of the parent hep-2A fragment. Subfragments containing intact module $III_{13}$ bind only 2–3-fold weaker, and a subfragment containing both modules $III_{13}$ and $III_{14}$ has an affinity indistinguishable from that of the parent, confirming by direct measurement the conclusion of Barkolow and Schwarzbauer (1991) that both of these modules are required for full activity. The results taken together with those obtained with synthetic peptides suggest that sites within and interactions between these two modules are important for maintaining full heparin binding activity.

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